Biodegradation of Petroleum in the Marine Environment: Limiting Factors and Methods of Enhancement

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April 1986

Canadian Technical Report of Fisheries and Aquatic Sciences No. 1442

87-03398/

Canadian Technical Report of Hydrography and Ocean Sciences

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Canadian Technical Report of
Hydrography and Ocean Sciences NO. 1442

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BIODEGRADATION OF PETROLEUM IN THE MARINE ENVIRONMENT: LIMITING FACTORS AND METHODS OF ENHANCEMENT

by

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Interdepartmental Panel on
Energy Research and Development (PERD)

Natural Degradation of Oil
O.E.R.D. Project Number: 67223

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Cat. No. FS 97-6/1442E ISSN 0706-6457

Correct citation for this publication:

Lee, K. and Levy, E.M. 1986. Biodegradation of petroleum in the marine environment: Limiting factors and methods of enhancement. Can. Tech. Rep. Fish. Aquat. Sci. No. 1442: iv + 65 p.

ABSTRACT

Lee, K. and Levy, E.M. 1986. Biodegradation of petroleum in the marine environment: Limiting factors and methods of enhancement. Can. Tech. Rep. Fish. Aquat. Sci. No. 1442: iv + 65 p.

Hydrocarbons are metabolically degraded by numerous microorganisms which have been found to be ubiquitous throughout the world's oceans. Maximal biodegradation rates are rarely attained under natural environmental conditions, and extensive studies have concluded that the degradation of petroleum in the marine environment is limited primarily by the availability of oxygen, nitrogen and phosphorus. During the last decade, the scientific community has recognized that enhancing the rate of natural biodegradation through oxygenation, chemical dispersion, and nutrient enrichment, may be an effective countermeasure to contamination by oil. This review of the existing literature clearly indicates that nutrient enrichment with nitrogen and phosphorus compounds is the most promising of the existing methods for enhancing biodegradation on a scale sufficient to afford a practical approach to combatting the problem of spilled oil.

RESUME

Lee, K. and Levy, E.M. 1986. Biodegradation of petroleum in the marine environment: Limiting factors and methods of enhancement. Can. Tech. Rep. Fish. Aquat. Sci. No. 1442: iv + 65 p.

Les océans du monde comptent de nombreux microorganismes qui provoquent la dégradation des hydrocarbures. Or, le taux de biodégradation maximum est rarement atteint dans des conditions environnementales naturelles, et des études approfondies révèlent que la dégradation du pétrole dans le milieu marin est limitée principalement par la disponibilité d'oxygène, d'azote et de phosphore. Au cours de la dernière décennie, le monde scientifique reconnaît que le fait d'augmenter le taux de biodégradation au moyen de l'oxygénation, de l'utilisation de produits chimiques dispersants et de l'introduction de nutrients peut lutter efficacement contre la polution par les hydrocarbures. Cet examen de la documentation actuelle indique clairement que l'introduction d'éléments nutritifs tels que des composés azotés et phosphoreux est la plus prometteuse des procédés actuellement employés pour favoriser la biodégradation dans une mesure suffisante pour constituer une méthode pratique de lutte contre le problème des déversements de pétrole.

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1.0. INTRODUCTION

The attention of the scientific community was dramatically focussed on the problems of oil pollution following the sinking of the supertanker "Torry Canyon" in 1967. While such accidents draw the public's interest, the inevitable losses of oil during routine operations of recovery, transportation and refining are considered to be major contributors to the total oil pollution problem. The National Academy of Sciences (1975), has estimated that over 6 million tonnes of oil, in both crude and refined form, enter the marine environment per year. Since the oceans appear to be the major receptient of hydrocarbon pollutants, scientific research has been focused on the transport and fate of oil in the marine environment (Wolfe, 1985; Atlas, 1981; Jordan and Payne, 1980; Colwell and Walker, 1977; Floodgate, 1984; National Academy of Sciences, 1985, 1975). In response to our ever increasing need for energy, petroleum development has expanded into new frontiers, including deep offshore and arctic waters. To protect these environments, research on the fate and effects of petroleum contaminants in the marine ecosystem must be maintained as a priority.

Changes which occur in the composition of petroleum exposed to the environment are known collectively as "weathering". When crude oil is spilled in an aquatic environment, evaporation generally removes components with boiling points of less than 370^{o}C from the system in a matter of days, leaving autocatalytic and biological decomposition to operate upon the remaining

fraction (Waite, 1984). Microbial biodegradation plays a major role in natural weathering of petroleum hydrocarbons (National Academy of Sciences, 1985; Atlas, 1981). The evolution of a hydrocarbon mixture in the environment thus depends on the nature of the oil, a variety of environmental factors, and the biota present.

The predominant microbial responses observed after an oil spill are: (1) the inhibition of specific naturally-occurring groups of bacteria that may be critical for maintaining the ecosystem in balance and, (2) the development of populations that are capable of biodegrading the oil. This review describes the major factors which influence the interaction between petroleum-degrading microorganisms and hydrocarbons in the marine environment. Furthermore, a synopsis of the techniques which have been developed and applied to enhance microbial degradation of hydrocarbons in the marine environment is provided.

2.0. HYDROCARBON-DEGRADING MICROORGANISMS

Zobell (1946) reported that numerous microorganisms have the capability to use hydrocarbons as their sole source of carbon and energy and that such microorganisms are widely distributed among diverse microbial populations. Since hydrocarbons are naturally-occurring organic compounds, as well as anthropogenic contaminants, it is not surprising that microorganisms have adapted themselves to metabolize these compounds. Hence, natural

ecosystems contaminated with petroleum are likely to contain indigenous microbial species which are capable of degrading the contaminating hydrocarbons.

The mechanisms of hydrocarbon uptake and metabolism by microorganisms have been well described in earlier publications (Singer and Finnerty, 1984; Cerniglia, 1984; Perry, 1984, 1979, 1977; Gibson, 1977, 1971; Pirnik, 1977; Gutnik and Rosenberg, 1977; Ratledge, 1978; Van der Linden and Thijsse, 1965; McKenna and Kalio, 1965; Foster 1962). Normal and branched-chain aliphatic hydrocarbons are typically oxidized at a terminal carbon, with the successive generation of primary alcohols, aldehydes, and fatty acids, which enter normal biochemical pathways. Aromatic ring structures are oxidized by mixed function oxidases to dihydroxy-substituted aromatic compounds, then the ring is cleaved to either a dicarboxylic acid or an aldehyde-carboxylic acid. Larger, condensed poly-aromatic hydrocarbons are metabolized through successive opening of the aromatic rings.

The environmental role of microorganisms in mitigating the impact of petroleum spillages upon the environment through biodegradative removal of contaminant hydrocarbons has been the subject of several reviews (Atlas, 1985a, 1981; Bossert and Bartha, 1984; Colwell and Walker, 1977; Floodgate, 1984; Zobell, 1964; National Academy of Sciences, 1975; Karrick, 1977; Van der Linden, 1978; Jordan and Payne, 1980). The hydrocarbons degraded

by microorganisms can be incompletely metabolized to compounds of lower carbon number, incorporated into additional cell-mass through normal growth-processes, or completely metabolized to end-products such as carbon dioxide, water and ammonia. It is important to emphasize that biodegradation usually does not mineralize all of the constituents in a complex hydrocarbon mixture such as crude oil. In a typical biodegradation sequence, straight-chain alkanes from C-10 to C-25 are rapidly degraded, followed by branched-chain alkanes such as pristane, thus leaving an unresolved complex mixture containing high molecular weight cyclic and aromatic compounds (Wong et al., 1984). However, many of the major residues resulting from microbial attack on crude oils are relatively harmless because of their low biological activity (Hughes and Stafford, 1983).

2.1. Species Involved

More than 100 species of microorganisms, representing 30 genera, were identified by Zobell (1946), to have the capability to metabolize petroleum. Mulkins-Phillips and Stewart (1974c), reported finding hydrocarbon-degrading bacteria of the genera Nocardia, Pseudomonas, Flavobacterium, Vibrio, and Achromobacter, in coastal waters and sediments from the northwest Atlantic. Bartha and Atlas (1977), isolated 22 genera of bacteria, 1 algal genus, and 14 genera of fungi which had the ability to use petroleum from aquatic environments. Based on the frequency of isolation, the predominant genera of hydrocarbon degrading

microorganisms were Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula and Sporobolomyces.

Ahearn et al. (1971), reported that some yeasts were capable of growing in seawater which contained 4% oil. Cerniglia and Perry (1973), reported that several fungi (Pencillium and Cunninghanella spp.) exhibited greater hydrocarbon biodegradation rates than bacteria (Flavobacterium, Brevibacterium and Arthrobacter spp.). Walker et al. (1975c) compared the abilities of bacteria and fungi to degrade hydrocarbons. From the study of the genera Candida, Sporobolomyces, Hansenula, Aureobasidium, Rhodotorula, Cladosporium, Penicillium, Aspergillus, Pseudomonas, Vibrio, Acinetobacter, Leucothrix, Nocardia and Rhizobium, bacteria and yeasts were found to have a decreasing ability to degrade alkanes with increasing chain length. In contrast, filamentous fungi did not exhibit preferential degradation of particular chain lengths within the alkane fraction.

Although specific strains of both yeasts and filamentous fungi may be very active in degrading hydrocarbons, their scarcity in the open ocean suggests that, in global terms, their activity may be insignificant. However, in inshore waters, on beaches, in salt marshes, and in sand dunes, their biomass and their local importance as degraders of oil increases (Floodgate, 1984).

Some cyanobacteria and algae have been reported to be capable of

metabolizing hydrocarbons. The most remarkable algal activity was reported by Walker et al. (1975a, 1976b), who found that a strain of the achlorophyllous alga Prototheca zopfi isolated from polluted sediments collected from Baltimore Harbour, was capable of degrading 10% of a motor oil, and 40% of a crude oil; a degradative capability comparable to that of bacteria. Cerniglia et al. (1980), found that cyanobacteria and algae such as Oscillatoria spp., Microcoleus sp., Anabaena spp., Agmenellum sp., Coccochloris sp., Nostoc sp., Aphanocapsa sp., Chlorella spp., Dunaliella sp., Chlamydomonas sp., Ulva sp., Cylindretheca sp., Amphora sp., Porphyridium sp., and Petalonia sp., were capable of producing a number of metabolites from naphthalene. Since only 19 cultures were assessed, the ability to oxidize aromatic hydrocarbons may be widely distributed among cyanobacteria and algae.

In summary, biodegradation of petroleum is not restricted to a few microbial genera; a diverse group of bacteria, fungi, yeasts and algae from marine, freshwater and soil habitats have been shown to have this ability (National Academy of Sciences, 1985; Austin et al., 1977). No single microbial species appears to be able to completely degrade any given oil. Microorganisms are selective, and many different microbial species in a mixed culture may be required for significant degradation. Hence microbial succession may be a significant factor in the degradation of petroleum in the marine environment.

2.2. Geographical Distribution

Petroleum degrading microorganisms appear to be ubiquitously distributed in the marine environment (Atlas, 1978; Robertson et al., 1973; Tagger et al., 1976; Walker and Colwell, 1976b; Mulkins-Phillips and Stewart, 1974c). However, due to the different techniques which have been used to enumerate potential oil-degrading microorganisms, their geographical distribution is not well known.

Bunch and Harland (1976), found the numbers of oleoclastic (petroleum degrading) microorganisms occurring in regions of the Beaufort Sea were comparable to those found in marine samples from subarctic Canadian waters. These results confirmed the presence of hydrocarbon-degrading microorganisms in the arctic marine ecosystem and suggest that quantitative differences in their distribution were relatively unimportant over large geographical areas. Similar results were found by Roubal and Atlas (1978) using the most probable number (MPN) procedure to determine the distribution of hydrocarbon-degrading microorganisms on the Alaskan Continental Shelf. They concluded that hydrocarbon degrading microorganisms were ubiquitous, with no significant overall differences in concentration between surface water and sediment samples in arctic and subarctic sampling regions.

Mulkins-Phillips and Stewart (1974c), examined the distribution of oleoclastic bacteria in northwestern Atlantic waters and

coastal sediments. The fraction of the total heterotrophic bacteria represented by the hydrocarbon-degraders ranged up to 100%, depending on the previous history of oil spillage in the region; most values were less than 10%. LePetit et al. (1977), reported that in a region contaminated by refinery effluent, bacteria capable of using a gas-oil mixture as their sole carbon source represented 10% of the total heterotrophic bacteria population, while in a area not obviously polluted by hydrocarbons the numbers of oleoclastic bacteria only accounted for 4% of the heterotrophic population. Degradation potential was found to be highest in areas of chronic discharge (Tagger et al., 1979). Gunkel et al. (1980), found high numbers of oleoclastic microorganisms in the vicinity of the North Sea oil fields and showed that there was a high correlation between hydrocarbons and oil-degrading bacteria in that area of the North Sea. The numbers and variety of the oleoclastic microbes found in diverse marine environments demonstrate their ubiquity and potential for removing or converting petroleum hydrocarbon contaminants.

The rapid rise in the numbers of hydrocarbon-degrading microorganisms following oil spills has been observed in several studies. Kator and Herwig (1977), found that within a few days of a spill of South Louisiana crude oil in a coastal estuary in Virginia, levels of petroleum-degrading bacteria rose by several orders of magnitude. These elevated numbers of hydrocarbon-degrading bacteria were sustained for over a year. Significantly

elevated numbers of oleoclastic microorganisms (orders of magnitude above the normal baseline levels), were also observed following the Amoco Cadiz spill in Brittany (Atlas and Bronner, 1981), and the XTOC-I well blowout in the Bay of Campeche, Gulf of Mexico (Atlas et al., 1980a, 1980b). In contrast, Bunch et al. (1983b), suggested that the number of total viable heterotrophs or the relative abundance of oil-degrading bacteria in the microbial community of the water column may not always increase in response to short-term (acute) exposure to high levels of oil. Microbial populations may decline in the presence of oil, especially if no induction of oil-degrading enzymes by prior exposure to hydrocarbons has occurred (Atlas and Bartha 1973b).

Numbers of hydrocarbon-degrading microorganisms are generally very low in areas of the open ocean away from sources of petroleum. However, along shipping lanes (Mironov, 1970), and in petroleum-polluted coastal waters (Buckley et al., 1976; Oppenheimer et al., 1977), population levels increase. Laboratory studies have conclusively demonstrated that numbers of hydrocarbon-degrading microorganisms within environmental samples generally increase in response to petroleum hydrocarbon exposure (Zobell, 1973; Soli, 1973; Colwell and Walker, 1977). Populations of hydrocarbon-degrading microorganisms generally increase in both the sediments and water of pristine environments exposed to petroleum, in both experimental (Atlas et al., 1978) and accidental spills (Colwell et al., 1978; Ward et al., 1980; Atlas and Bronner, 1981). It is now clear from a number of

studies that the distribution of hydrocarbon-utilizing microorganisms quantitatively reflects the degree or extent of exposure of the ecosystem to hydrocarbon contaminants. In unpolluted ecosystems, oleoclastic microorganisms generally constitute less than 0.1% of the microbial community; in hydrocarbon-contaminated ecosystems, they can constitute up to 100% of the viable microorganisms (Azoulay et al., 1983; Atlas, 1981).

2.3. Potential Biodegradation Rates

The biodegradation rate of hydrocarbons in the marine environment is highly dependent on the composition of the biota, the chemical nature of the petroleum mixture, and environmental conditions. Current data indicates that potential hydrocarbon-degrading activity, as measured by ^{14}C hydrocarbon uptake and mineralization studies, does not correlate with the size of the total heterotrophic population but rather with the number of hydrocarbon degrading microorganisms.

Zobell (1969), estimated that the degradation rates of crude oils, lubricating oils, cutting oils and oil wastes ranged from 0.02 to 2.0 $g/m^2/day$ at temperatures between 24 and 30°C. Atlas and Bronner (1981), estimated that microbial degradation could remove up to 0.5 kg of petroleum hydrocarbons $hectare^{-1}$ day^{-1} from intertidal sediments. Arhelger et al. (1977), studied petroleum biodegradation in the Arctic and Subarctic and reported

the following biodegradation rate: in Port Valdez - 0.7 g/liter per day; in Chukchi Sea - 0.5 g/liter/day; and in the Arctic Ocean - 0.001 g/liter/day. The observed rates of hydrocarbon degradation showed a definite link to climatic conditions. The rates were found to be lower in the Arctic Ocean than in the more southerly Alaskan Waters. From their experiments conducted in Arctic and subarctic Alaskan waters, Roubal and Atlas (1978), concluded that there was no significant correlation between the numbers of oleoclastic microorganisms and the hydrocarbon biodegradation potentials. In general, arctic and subarctic studies of experimental oil releases and accidential spillages report low rates of microbial degradation and long persistence times for the hydrocarbon contaminants.

Hydrocarbon biodegradation rates in the marine environment reported thus far have generally been estimated by measuring the amount of oil at the beginning and the end of an experiment, and are based on the unsupported assumption that the rates are linear and similar for all components.

Walker et al. (1976b), monitored the fate of individual fractions of South Louisiana crude oil exposed to estuarine sediments. As a percent of the weathered oil remaining, saturates were the only components which showed a consistent decline during the 7 week experimental period. The percentage of aromatics and resins, comprising of pyridines, quinolenes, carbazoles, sulfoxides and amides initially declined, then increased in the latter part of

the experiment (3rd to 7th week). Based on the results of radioisotope studies, Walker and Colwell (1976b) reported that the mineralization rate for hexadecane was greater than that for naphthalene, which was greater than those for toluene and cyclohexane. In a similar study, Lee (1977b), noted that alkanes and low molecular-weight aromatics (benzene, toluene, naphthalene and methyl-naphthalene) were degraded to CO_2 by microorganisms present in riverwater, but higher molecular-weight aromatics were relatively resistant to microbial degradation. It is now widely accepted that while biodegradation of different classes of petroleum compounds occurs simultaneously, but they occur at different rates. In the marine environment, studies generally indicate that normal alkanes are degraded first, followed by iso-alkanes, cycloparaffins, aromatics, and larger polycyclic aromatics (Bartha and Atlas, 1977).

3.0. FACTORS INFLUENCING BIODEGRADATION

The persistence of petroleum contaminants within the marine environment is dependent on a combination of factors which include the physical/chemical properties of seawater and oil, environmental conditions and the biota. Due to the multitude of interactions between these factors and the different rates of metabolism for the various components within petroleum, it is difficult to predict accurately the biodegradation rates of petroleum in the environment. However, the potential effect of these factors has been examined individually, and the general

direction in which they will alter the oil can be predicted qualitatively.

3.1. Biota

Populations of microorganisms found in an oil contaminated environment will degrade petroleum differently and at a different rate than microorganisms present in a pristine environment. Indigeneous microbial flora respond to elevated oil concentrations by increasing their oil-degradative capacity, either by selectively promoting the growth of oil-degrading strains and/or by inducing enzymatic mechanisms within the existing strains. At sites where oil is already present, either from acute accidental spills or from chronic long term seepages, there will be greater numbers of oil degrading bacteria presumably because of continuous enrichment (Zobell and Prokop, 1966; Roubal and Atlas, 1970; Walker and Colwell, 1974; Gunkel and Gassman, 1980). At such oil-enriched sites, the degradation of newly-introduced oil may occurred much more rapidly (Colwell and Walker, 1977).

The potential hydrocarbon degrading activity of microbial populations has been quantified by numerous investigators using ^{14}C-radiolabelled hydrocarbons. Caparello and LaRock (1975), found that the hydrocarbon-oxidizing potential in natural waters and sediments reflected the hydrocarbon burden of the area sampled and the ability of the indigeneous microorganisms to

metabolize hydrocarbons. Walker and Colwell (1976a), reported higher rates of uptake and mineralization in samples collected from an oil polluted harbour in comparison to similar samples from a relatively unpolluted region. They reported turnover times of 15 and 60 minutes for the polluted and unpolluted areas respectively, using ^{14}C-hexadecane. It must be emphasized however, that the "heterotrophic potential" technique, based on the uptake and/or mineralization of ^{14}C-labelled substrates, is only a relative index of bacterial activity to specific substrates and not an absolute measure of heterotrophic production and total oil degradation.

During a model oil spill study in Arctic coastal waters, using chromatographic-mass spectral analysis, Horowitz and Atlas (1977a), found that regardless of the amount of degradation, the percentages of the individual components within the spilled petroleum mixture were not altered. This indicated that most hydrocarbon components of the oil were degraded at similar rates. Co-metabolism, in which normally resistant compounds within the petroleum mixture are enzymatically attacked by microorganisms which were sustained on other organic substrates (Horvath, 1972; Perry, 1979; Cerniglia et al, 1980; Gibson, 1977), was hypothesized to be responsible. The numerous potential substrates within petroleum hydrocarbon mixtures provide an ideal chemical environment in which co-oxidation processes can occur. Many complex branched and cyclic hydrocarbon contaminants in the environment are undoubtedly removed as a result of co-oxidation processes (Perry, 1977; Raymond and Jamison, 1971). However, the

ecological significance of co-metabolism in the natural environment is difficult to ascertain since multiple species are present.

While the mechanism of diauxic growth does not alter the metabolic pathways of degradation, it does control the time at which the enzyme necessary for metabolic attack of a particular hydrocarbon is produced or activated. Pirnik et al. (1974), observed a diauxic phenomenon for the degradation of pristane, in which pristane was not degraded in the presence of hexadecane. Horowitz et al. (1975), used sequential enrichment to isolate organisms which could metabolize progressively more complex (i.e., resistant to microbial degradation) compounds. Sparing effects also alter the persistence of particular hydrocarbons within a petroleum mixture. For example, LePetit and Tagger (1976), reported that acetate, an intermediate product in hydrocarbon biodegradation, reduced the utilization of hexadecane.

3.2. Physical / Chemical Properties of Oil

Differences in the composition and concentration of petroleum hydrocarbons influence their susceptibility to biodegradation. For example, Mulkins-Phillips and Stewart (1974a), reported that under similar experimental conditions, a Nocardia sp. degraded 94% of the n-alkanes and 35% of the unresolved fraction of an Arabian crude oil, while only 77 and 13% were lost respectively,

in a Venezuelan crude. Walker et al. (1976a), monitored the biodegradation of two crude oils (South Louisiana and Kuwait) and two refined fuel oils (#2 and #6) by indigeneous bacteria within Baltimore Harbour sediments. Gravimetric analysis after 7 weeks indicated that the relative biodegradability of the crude oils differed, with 22% of the South Louisiana crude oil remaining, 49% of the Kuiwait crude oil, 45% of the No. 2 Fuel Oil and 89% of the No. 6 Fuel Oil (Bunker C). Corresponding analyses of samples by column chromatography and mass spectrometry indicated that some components of the petroleum were selectively degraded while others persisted. Furthermore, in support of the results obtained by Mulkins-Phillips and Stewart (1974a), they also reported significant differences in the susceptibility of identical compounds within the the different hydrocarbon mixtures in the oils tested to degradation.

Changes in the biodegradation rate of oil spilled in the marine environment may occur due to alterations in its physical state. For example, the formation of mousse and tar balls inhibits the biodegradation and abiotic weathering of oil by restricting the availability of oxygen and mineral nutrients, and by reducing photooxidative and evaporative losses (National Academy of Sciences, 1985). Such interactive processes have been suggested as major limiting factors for petroleum biodegradation at the Ixtoc-I (Atlas et al., 1980a) and Metula (Colwell et al., 1978) oil spills.

In general, the rate of oil loss via biodegradation processes tends to become slower as recalcitant substances form a greater proportion of the residue. Low biodegradation rates are found when the substrate contains a high proportion of condensed polyaromatic compounds, condensed cycloparaffins, asphaltenes, and resin compounds, while a high proportion of medium length n-alkanes makes for a quicker loss.

3.3. Temperature

Psychrotrophic, mesophilic, and thermophilic hydrocarbonoclastic microorganisms have been isolated from the environment and microbial biodegradation of petroleum hydrocarbons has been observed at temperatures ranging from below 0°C (Zobell, 1973a; Traxler, 1973) to above 70°C (Mateles et al., 1967).

In addition to having direct effects on the biota, temperature changes also alter the physical/chemical state of petroleum hydrocarbons within the environment. Low temperature decreases the rates of volatilization and increases the solubility of low-molecular weight hydrocarbons, some of which are toxic to microflora (Atlas, 1975; Atlas and Bartha, 1972a; Walker and Colwell, 1974). Consequently, in an oil spill under low temperature conditions, one would expect the onset of biodegradation to be delayed because the toxic fractions of the hydrocarbons are sustained within the water column for a longer period of time. This hypothesis was confirmed by Atlas (1975),

who examined the effects of temperature on the biodegradability of seven different crude oils. At 20°C, lighter oils had greater abiotic losses and were more susceptible to biodegradation than heavier oils. At 10°C, the toxic volatile components of light crude oils evaporated more slowly, causing a significant lag before the onset of hydrocarbon degradation. Toxic volatile fractions were not observed in the heavy oil samples tested. Some indication of a preference for paraffin degradation appeared at lower temperatures.

The combined impact of temperature and the physical/chemical state of the petroleum hydrocarbons on biodegradation was well illustrated by Atlas (1981). The alkane, hexadecane, common to crude oils only supported marginal bacterial growth at 5°C when the compound was in solid form, but when it was dissolved in another liquid hydrocarbon or crude oil at the same temperature, extensive degradation was observed.

While hydrocarbons are somewhat soluble in water, oil spill incidents generally involve concentrations far in excess of the solubility limits. The degree of spreading or slick formation, which determines in part the surface area of the oil available for colonization by hydrocarbon degrading microorganisms, is reduced by the low temperature related changes in the viscosity of the oil.

The influence of temperature and the chemical composition of crude oils on the selection of hydrocarbon-degrading

microorganisms were described by Cook and Westlake (1974). At 4°C, Achromobacter, Alcaligenes, Flavobacterium, and Cytophaga were isolated on a substrate of Prudhoe Bay crude oil; Acinetobacter, Pseudomanas, and unidentified gram-negative cocci on Atkinson Point crude oil; and Flavobacterium, Cytophaga, Pseudomanas, and Xanthomonas on Norman Wells crude oil. At 30°C, substantially different major genera were isolated on the various crude oils. On Prudhoe Bay crude oil the major genera were Achromobacter, Arthrobacter, and Pseudomanas; on Atkinson Point crude oil, they were Achromobacter, Alcaligenes, and Xanthomonas; and on Norman Wells crude oil, they were Acinetobacter, Arthrobacter, Xanthomonas, and other gram negative rods.

Environmental adaptation by oil degrading microbes to low temperature conditions is well documented by Colwell et al. (1978). They reported greater degradation of Metula crude oil at 3°C than at 22°C by indigeneous populations of microorganisms found in beach sand collected from the Antarctic ecosystem. In laboratory studies at 0.1% oil concentration, with Antarctic isolates adapted to the incubation temperatures, 48% of the added hydrocarbons were degraded at 3°C, compared to 21% at 22°C.

Extensive research on the biodegradation of Prudhoe Bay crude oil in the Arctic coastal ecosystem have been conducted (Atlas, 1985, 1977, 1978; Atlas et al., 1978). Biodegradation potentials for Prudhoe Bay crude oil were much lower in ice than in water or sediment. Natural rates of biodegradation were found to be slow

during an Arctic Summer period with abiotic and biodegradative processes accounting for less than 50% of the oil loss. In the arctic environment, microbial degradation involving indigenous psychrophilic and psychrotrophic microorganisms will ensue, albeit much more slowly than during a warm season in a temperate zone. Rates of oil biodegradation in the Arctic appear to be limited by temperature-dependent factors (e.g. physical state of the oil and ice conditions) and the concentration of available nutrients.

Changes in temperature have also been correlated with the induction and/or suppression of metabolic pathways within microorganisms. In contrast to most studies, Horowitz and Atlas (1977a), found that different structural classes of hydrocarbons were degraded at similar rates, in arctic surface waters during the summer. They postulated that co-metabolism, resulting from the induction of enzymes that used both growth supporting substrates and their less useful analogues was enhanced by low temperatures.

3.4. Nutrients

All seawater samples appear to contain bacteria capable of oxidizing some of the multitudinous components of petroleum. The sea, however, is far from being an ideal medium for bacterial growth. Although the total concentration of organic substances is high, the concentration of nutrients necessary for

heterotrophic processes is maintained low by competition between microbial species including phytoplankton. Nitrogen and phosphorus are the most probable nutrients which limit microbial degradation of hydrocarbons in the sea (Gibbs and Davis, 1976; Bergstein and Vestal, 1978; Horowitz and Atlas, 1978). Of the two nutrients, phosphorus is less likely to be the limiting nutrient since it appears to be rapidly recycled (Riley and Chester, 1971).

As the result of studies on the effects of nutrient limitation on the biodegradation of hydrocarbons in the oceans, Floodgate (1979) proposed the concept of "nitrogen demand", analogous to the concept of the biochemical oxygen demand. Based on the biodegradation of Kuwait oil at 14°C, the nitrogen demand was found to be 0.4 umol of nitrogen per mg of oil. This concept was subsequently utilized to estimate the biodegradation rate of oil in the environment. Following the sinking of the supertanker Amoco Cadiz off the coast of Brittany, Aminot (1980), observed a depletion of N, P, and O_2 in the water column beneath the oil slick as the result of microbial degradation of petroleum. Calculations from the in situ deficits of N, P, and O_2 indicated that 9,000 metric tons of oil was biodegraded within the water column during the 2 week period following the spill.

Laboratory studies conducted under simulated field conditions have provided convincing evidence which shows that the slow rates of petroleum degradation often observed in the marine environment are attributable to nutrient limitation. Atlas and Bartha

(1972b, 1973c), reported an eight to nine fold increase in the extent of oil biodegradation in flow-through sea water tanks with oil-water mixtures when nitrogen and phosphorus salts or a combination of octyl-phosphate and a slow-release paraffinized urea fertilizer was added. Walker and Colwell (1975), and Walker el al. (1976c), also reported more extensive degradation of mixed hydrocarbon substrates in batch cultures of marine bacteria in seawater supplemented with inorganic nitrogen.

Experiments have been conducted to determine the optimal nutrient concentrations required to support maximal biodegradation rates of petroleum hydrocarbons in the marine environment. Bridie and Bos (1971), reported that the addition of 3.2 mg of ammonia nitrogen and 0.6 mg of phosphate maintained maximum degradation rates for Kuwait crude oil at a concentration of 70 mg of oil per liter. Atlas and Bartha (1972b), found that concentrations of 1.0 mg of nitrogen and 0.7 mg of phosphorus per liter supported maximum degradation of Sweden crude oil (8 g per liter) in New Jersey coastal seawaters. For the degradation of Iranian crude oil in Mediterranean seawater at a concentration of 1 g per liter, Reisfeld et al. (1972), reported that the optimal concentrations of nitrogen and phosphorus were 11 and 2 mg per liter, respectively.

Nutrient concentrations also appear to be a predominant factor controlling hydrocarbon biodegradation in the Arctic. Haines and Atlas (1982), reported that biodegradation of Prudhoe Bay crude

oil in nearshore sediments of the Beauford Sea was only apparent after one year of exposure. This initial lack of response by the microbial community was not attributed directly to low temperatures, since psychrotrophic or psychrophilic hydrocarbonoclastic microorganisms are capable of active growth and metabolism at temperatures below 0°C (Traxler, 1973; Zobell, 1973a; Robertson et al., 1973; Morita, 1975). Nutrient and oxygen concentrations were hypothesized to be the limiting factors, since Prudhoe Bay crude oil under similar temperature conditions, was previously reported not to contain components which were inhibitory to microbial hydrocarbon degradation (Atlas, 1975). Assuming that the optimal rates of hydrocarbon biodegradation occur at C:N and C:P ratios of 10:1 and 30:1 (Atlas and Bartha, 1972b), Arctic sediments would have required several orders of magnitude higher concentrations of N and P to support maximal rates of hydrocarbon biodegradation.

Horowitz and Atlas (1977a), observed large increases in bacterial numbers and corresponding shifts in population structure to hydrocarbon utilizers in Arctic coastal waters when nitrogen and phosphorus were added to oil slicks. Bergstein and Vestal (1978), reported the lack of elevated microbial populations in an oil-treated tundra pond unless phosphate was also added. Following a spill in the Antarctic marine environment, Colwell et al. (1978), concluded that Metula oil was degraded slowly because of limitations imposed by the relatively low concentrations of nitrogen and phosphorus available in the seawater.

3.5. Oxygen

The oxygen requirement for bacterial degradation of oil can be substantial. Zobell (1969), estimated that 3 to 4 milligrams of dissolved oxygen were necessary to convert one milliliter of hydrocarbon into carbon dioxide and water. In the open ocean, oxygen is not usually considered to be a limiting factor for the degradation of hydrocarbons, since the upper parts of the water column where oil is most likely to be found, is usually well oxygenated. Furthermore, the Michaelis constant of the oxygenase enzymes responsible for the biodegradation of oil have a high affinity for oxygen and thus will function even when the concentration of dissolved oxygen is low. (Floodgate, 1976b). However, Aminot (1981), reported that some microbial populations in areas polluted by the grounding of the Amoco Cadiz were oxygen, as well as, nitrogen and phosphorus limited.

Low energy beaches and sediments characterized by silts and muds are often reported to be oxygen depleted (Colwell and Walker, 1977; Gibbs and Davis, 1976; Ward et al., 1980). Johnson (1970) showed that the oxygen concentration of interstitial waters within laboratory sand columns decreased rapidly in response to Kuwait crude oil. The mean rate of oxygen consumption over 4 months was 0.45 g/m^2 per day at $10^{o}C$, corresponding to an oil degradation rate of 90 mg of oil/m^2 per day.

There have been reports of isolated microorganisms capable of degrading hydrocarbons, especially the n-alkanes, under anaerobic

conditions (Chouteau et al., 1962; Parekh et al., 1977; Senez and Azoulay, 1961; Traxler and Bernard, 1969; Iizuka et al., 1969), as well as preliminary reports of anaerobic hydrocarbon degradation in natural ecosystems (Bailey et al., 1973; Brown et al., 1969; Pierce et al., 1975; Zobell and Prokop, 1966).

Ward et al. (1980), compared the rates of hydrocarbon oxidation in sediments affected by the Amoco Cadiz spillage under both aerobic and anaerobic conditions. With ^{14}C-labelled hydrocarbons, under strict anaerobic conditions in which methanogenesis was evident, $^{14}CO_2$ production was observed from heptadecane and toluene, but not from hexadecane. Rates of $^{14}CO_2$ production were orders of magnitude higher under aerobic conditions than under anaerobic conditions. In the absence of oxygen, less than 5% of the added saturate and aromatic hydrocarbons were oxidized to $^{14}CO_2$ in 233 days compared with the 47% in 14 days under aerobic conditions. Chemical analyses of anaerobic sediments oiled by the Amoco Cadiz spill suggest that hydrocarbons in the environment were protected from microbial attack under anaerobic conditions. Subsequent studies also suggest that anaerobic biodegradation of hydrocarbons in the natural environment proceeds at negligible rates.

Conditions which enhance aeration and replenishment of nutrients, favour biodegradation. For example, Rashid (1974) after a 3 year study, concluded that the degradation of oil spilled on the coast of Nova Scotia from the grounding of the tanker Arrow depended

largely on environmental factors, such as wave energy. Degradation was greatest in high wave-energy environments and lowest in protected embayment areas. In high-wave energy environments, there was a preferential loss of n-alkanes, which was attributed to microbial degradation. Presumably, oxygen and nutrient replenishment by wave-driven mixing permitted more extensive degradation. Biodegradation of oil in sediments has been found to be stimulated by bioturbation (Gordon et al., 1978; Lee, 1977), through the introduction of oxygen into oil-contaminated sediments by burrowing animals such as polychaete worms.

3.6. Salinity / Pressure

The salinity values observed in the world's oceans are easily within the range tolerated by petroleum-degrading bacteria. While estuaries present a salinity, nutrient, and oxygen regime that is very different from either coastal or oceanic areas, they are not inhibitory to microbial activity. Hence, studies on the effects of salinity on microbial biodegradation of hydrocarbons have been primarily concerned with hypersaline environments (Ward and Brock, 1978)

In addition to hydrocarbons originating from deep water seeps, hydrocarbon contaminants from the ocean surface enter the sediments of ocean basins as a result of weathering processes which alter their specific gravity. Microbial degradation of

organic matter in the deep sea is greatly restricted (Jannasch et al. (1971), and hydrocarbons do not appear to be an exception. Using ^{14}C-labelled hydrocarbons in a mixed hydrocarbon substrate, Schwartz et al. (1974a, 1974b, 1975), found that at 1 atm pressure and 4°C, maximum growth by the bacteria on hexadecane was reached within 4 weeks, while at 500 atm pressure, maximum growth required 32 weeks incubation. Furthermore, while biodegradation of 94% of the hexadecane required 8 weeks at 1 atm pressure, at 500 atm pressure 40 weeks was required. These results indicate that oil entering deep-ocean environments will be degraded very slowly and persist for long periods of time.

ENHANCEMENT OF MICROBIAL DEGRADATION

Biodegradation of oil in the marine environment is a natural, generally slow, process. According to Miget (1973), while regions polluted by oil spills are usually colonized by large populations of oil-degrading bacteria within one or two weeks, two months or more may be necessary before biodegradation is observed. Attempts to increase the rate of biodegradation of petroleum in the environment are based upon the modification of those factors which influence or limit the necessary metabolic processes. At present, however, our knowledge of the interactions, and/or the sequence of events, which occur during microbial degradation of hydrocarbons in the environment is incomplete.

Case histories of methods which have been developed to enhance the biodegradation of hydrocarbons in the field are summarized in this chapter. Although limited success has been achieved in the laboratory and at controlled field sites, most attempts in the open ocean have failed.

4.1. Oxygenation

Oxygen is not usually considered a limiting factor in the biodegradation of hydrocarbons in the open ocean since the upper part of the water column, where oil is most evident, is usually well-oxygenated by natural forces such as wind and wave action. In other regions, however, rates of petroleum biodegradation may be limited by oxygen availability, and biodegradation may be enhanced by the provision of more oxygen.

Jamison et al. (1975) used forced aeration to enhance hydrocarbon biodegradation in a groundwater supply which was contaminated by a gasoline spill. The addition of nutrients without aeration failed to stimulate biodegradation, but when both nutrients and oxygen were supplied, it was estimated that up to 1,000 barrels of gasoline were removed by microbial degradation.

Oil spills along the shoreline create oiled-wastes from the mixing of oil with inorganic and organic materials. Enhanced biodegradation was recognized by Halmo (1975), as one of the principal methods of treating oil-contaminated waste along the

shoreline. Oxygen was not considered to be the limiting factor for the biodegradation of oil standed on the shoreline. However, in composting studies, where oiled seaweed was mixed with oil sorbents and fertilizers, aeration was the primary limiting parameter. A simple system of forced aeration from the bottom of the windrows was more efficient and less labour intensive than weekly mechanical mixing. The porosity of the oily mixtures which controlled the degree of aeration was dependent on the type of oil sorbent used. The most effective combination was obtained by mixing the oily seaweed with pine bark and a nutrient mixture of urea in a microemulsion of oleic acid. Windrow composting of this mixture with forced aeration, resulted in approximately 65% of the paraffins in a weathered Statfjord crude oil being degraded within one summer, 70% over an entire year.

4.2. Seeding

Microbial seeding, as opposed to mechanical removal, has been considered as a method to "clean up" oil contaminated sites.

Although hydrocarbonoclastic bacteria with constitutive enzyme systems have been isolated, most of them use adaptive systems, which appear only after an extended period of contact with hydrocarbons. During this adaption period, chemical and physical reactions such as evaporation, auto-oxidation and photo-oxidation may produce components which are less easily metabolized or can even be toxic to bacteria. The addition of appropriate oil

degrading bacteria immediately following an oil spill has been suggested as a method of enhanced petroleum biodegradation during the early stages of a spill.

The observation that a large number of Pseudomanas had the capability to degrade hydrocarbons encouraged extensive study in genetics and enzymology with regard to hydrocarbon degradation (Chakrabarty, 1976, 1972; Chakrabarty et al., 1973; Williams, 1978). Following the discovery that the hydrocarbon degradation trait in these organisms was located in the plasmids, genetically-engineered bacteria which contained several plasmids each carrying genes for the degradation of a major class of petroleum hydrocarbons were developed (Friello et al., 1976) and patented (Diamond v. Chakrabarty, 1980).

Seeding of oil-contaminated environments with enriched cultures and/or genetically-engineered strains of bacteria was considered as a means by which the rate and extent of specific biodegradative processes in the environment could be enhanced (Liu and Dutka, 1972; LaRock and Severence, 1973; Reisfield et al., 1972; Friello et al., 1976; Weinberger and Kollenbrander, 1979; Atlas and Bartha, 1973a; Kado and Lui, 1981).

There have not been any large-scale in situ experiments to assess the effectiveness of seeding as a means of enhancing the biodegradation of petroleum hydrocarbons in the marine environment. On land, Jobson et al. (1974), found that the seeding of oil-contaminated soil with oil-degrading bacteria had

a much smaller effect in terms of enhanced degradation than the addition of fertilizers (i.e., nitrogen and phosphate). Westlake et al. (1978), in later studies within the boreal region of the Northwest Territories, also reported a rapid increase in bacterial numbers in oiled plots which had been treated with fertilizers containing nitrogen and phosphorus. This increase in bacterial numbers was followed by the rapid disappearance of n-alkanes and isoprenoids and a continuous loss of weight of saturated compounds in the recovered oil. In support of the previous study, no significant effect on the composition of the recovered oil was observed in the oil-contaminated plots seeded with hydrocarbonoclastic bacteria. In a model marine study conducted in $10m^3$ enclosures, Tagger et al. (1983), reported that the addition of a mixed culture of marine hydrocarbon-degrading bacteria to petroleum-polluted seawater did not increase its hydrocarbon degradation capability. All strains of the mixed culture disappeared from the dominant microflora while the autochthonous bacteria showed a capacity for adaptation to petroleum degradation approximately four days following the spill. In conclusion, treating the experimental spill by addition of a large quantity of a mixed culture of hydrocarbon-degrading bacteria did not appear to be effective; five months following the spill, the differences between the treated and untreated basins could not be detected by an uninformed observer.

Reisfield et al. (1972) observed that bacteria of the genus Arthrobacter appeared to be extremely efficient at emulsifying

petroleum hydrocarbons. They were able to isolate a strain capable of converting 65% of their test oil into substances which were not extractable by benzene. Following inoculation with Arthrobacter, oil in test enclosures was reported to be dispersed in less than one day. However, from the experimental evidence, it is apparent that these microorganisms may not degrade components within the oil, but simply convert it to emulsified forms which become resuspended in the water column.

No single microbial species appears to possess the enzymatic capacity to metabolize more than two or three of the components present in petroleum hydrocarbon mixtures. Many different bacterial species in mixed culture may be required for significant degradation of a petroleum hydrocarbon mixture such as crude oil to occur (Westlake, 1982). There is little evidence to indicate that improvements deriving from the addition of allochthonous microorganisms are sustained under natural conditions where they must compete for survival against the mixed population of natural bacteria (Tagger et al., 1983; Tramier and Siruois, 1983; Westlake et al., 1978). Natural bacterial populations have the inductive enzymatic abilities necessary for hydrocarbon degradation. The growth potential of bacteria is unimaginably great and the classic calculation, in which a single bacterium forms a mass the size of the earth after 100 generations (within 50 hours) is well known. Seeding of oil spills offers few advantages since oil degradation in the natural environment is not limited by the need for inocula. Microbial growth in the marine environment appears to be limited primarily

by abiotic factors, such as nutrient concentration. Studies on the natural dynamics of bacterial populations by Miget (1973) and Atlas and Bartha (1973a) have clearly demonstrated that when nitrogen and phosphorus concentrations limit bacterial growth following an oil spill, it is futile to treat it with additional bacterial species adapted to hydrocarbon degradation.

In conclusion, microbial seeding in the aquatic environment only offers promise in the treatment of contained spills or lagooned effluent where conditions can be more or less controlled. The open sea and the coastline during inclement weather offers a most unlikely situation for successful seeding of oil spills. Furthermore, seeding must be undertaken with precaution because of potential environmental impact, including potential pathogenicity to humans (Cobet et al., 1973).

4.3. Chemical Dispersion

Direct microscopic observations show that hydrocarbon-degrading microbes act mainly at the oil-water interface. Thus, biodegradation rates may be accelerated by chemical dispersants which increase the surface area of the oil-water interface by enhancing the rate at which oil droplets are sheared from the slick (Gatellier et al., 1973). Not only is the oil made more available to the microorganisms following dispersion but, as emulsified droplets move through the water column, oxygen and nutrients become more readily available to them.

There has long been a controversy regarding the use of chemical dispersants as a countermeasure to combat oil spills in the environment. This has largely been due to the results from early field trials such as the Torry Canyon incident (Smith, 1968), in which the solvents in the dispersant formulations caused greater ecological damage than the oil itself. The effects of chemical oil dispersants which we observe in the marine environment are the result of enhanced oxidation rates and loss of bacteria due to toxicity. Preliminary studies on the biodegradation of hydrocarbons have shown both stimulatory and inhibitory responses by the microbiota following the addition of chemical dispersants. For example, Mulkins-Phillips and Stewart (1974b), found that of four dispersants studied, only one (Sugee 2) increased the degradation of n-alkanes in crude oil. Gatellier et al. (1973) and Robichaux and Myrick (1972) likewise found that although some dispersants inhibited populations of hydrocarbon-oxidizing microorganisms, others enhanced them.

The second generation of chemical dispersants are much less toxic and more effective than the early formulations (Gerlach, 1981). However, they are not without biological effects (Lee et al., 1985; Hagstrom and Lonning, 1977; Hsiao et al., 1978; Rogerson and Berger, 1981). All dispersants can theoretically inhibit microbial activity by reducing the surface tension to levels which inhibit the growth of bacteria (Colwell and Walker, 1977; Blackman et al., 1978). Griffiths et al. (1981a, 1981b), has

reported that the rates of glucose uptake and mineralization in Arctic and Subarctic marine waters and sediments are suppressed by the presence of the dispersant Corexit 9527. Depressed glucose uptake rates were observed at concentrations as low as 1 ppm of Corexit. After studying the effects of weathered Lago Medio petroleum crude on bacteria from nearshore locations in the Northwest Territories of Canada, Bunch et al. (1983b), also concluded that the addition of Corexit to spilled petroleum crude may limit microbial activity. While the activity and numbers of oil-degrading bacteria did not appear to be affected by the oil additions alone, samples treated with Corexit 9527 in a concentration of 0.01%, consistently showed lower levels of glutamic acid and hexadecane mineralization than untreated samples.

The behaviour of dispersed oil has been studied in the laboratory and under oceanic conditions with a view to assessing the probability that toxic exposure conditions can be established within the water column. Following an oil spill, concentrations of oil in the mg/l range are believed to be achieved for a period of some hours under dispersed slicks (McAuliffe et al., 1980). Contradictory results have been obtained in biological studies. While the higher dilution rates of dispersed oil may reduce toxic effects, highly toxic oil components which normally evaporate from untreated oil may be dissolved in the water by the dispersant. In general, oil emulsions produced by dispersants are more toxic than either the crude oil or dispersant alone (Swedmark et al., 1973; Parsons et al., 1985). Furthermore,

since biodegradation rates of oil under the sea surface are generally slow, dispersed oil droplets may be deposited on the seafloor, particularly in shallow waters (Schwarz et al., 1974a).

Bunch et al. (1983a) reported that addition of the dispersant, Corexit 9527, severely reduced the mineralization of ^{14}C-hexadecane during a 60-day laboratory experiment. In particular, the dispersant was found to extend the lag prior to hexadecane mineralization. However, the addition of nitrate and phosphate immediately enhanced the rate of hexadecane mineralization in the samples. Similar observations were made by Foght and Westlake (1982). Enhanced growth of the microbial flora, in response to additional carbon input from Corexit, reduced nitrate and phosphate levels and delayed the oxidation of hydrocarbons. Components of the dispersant were preferentially used. Biodegradation of petroleum in marine environments containing low levels of nitrate and phosphate may be severely retarded by the addition of the dispersant. Recent studies by Lee et al. (1985), indicate that Corexit 9527, at concentrations similar to those expected in actual field applications, stimulated bacterial production by inducing the release of organic compounds from the indigeneous phytoplankton population, as well as directly serving as a growth substrate. Furthermore, Lee et al. (1985), also attributed the elevated bacterial standing stock in waters treated with dispersant to an acute toxic response by other organisms (the bacterivores) to the dispersant.

The use of chemical dispersants in the marine environment is complicated by the large number of factors which influence dispersant performance, including dispersant to oil volume ratio, temperature, salinity, sea state, physicochemical properties of the oil and the nature of the dispersant-oil mixing process (MacKay and Wells, 1983; MacKay 1981). As an oil slick spreads, weathers and takes up water to form a water-in-oil emulsion, its viscosity increases. MacKay (1985) has stated that when the viscosity exceeds 3000-8000 mPa, the required dispersant dosage becomes so high that dispersion is no longer practical. Hence there is a limited period of a few hours to several days, depending on the prevailing spill conditions, within which application of dispersants is effective. Recent statistical analysis of chemical dispersant field trials by Fingas (1985) indicate that their field effectiveness was very low (<33%). Consistently effective application of chemical dispersants was only observed in the field trials where the dispersants were premixed with the oil.

While chemical dispersants remove oil from the sea surface, and are thus effective from the cosmetic point of view, they do not eliminate the toxic components of oil. Chemical dispersants merely aid the transport of oil from the surface into the water column. Furthermore, in spite of the well-documented capacity of various chemical dispersants to reduce the size of oil particles and disperse them in the water column, there have been no conclusive studies which have shown that dispersants enhance the extent of biodegradation. All experiments conducted to date

suggest that while the rate of mineralization may increase in response to the addition of dispersants, refractory components of petroleum in the environment will persist regardless of the addition of dispersants.

4.4 Nutrient Enrichment

Growth kinetics of microorganisms in the marine environment often approach that of a chemostat in which growth is limited by deficiency in one of the nutrients. In oil-contaminated environments, carbon is greatly in excess and the limiting factors for *in situ* microbial degradation of petroleum in seawater are most likely to be the concentrations of nitrate and phosphate since petroleum hydrocarbons themselves contain virtually no nitrogen or phosphorus.

Preliminary laboratory tests (Van der Linden, 1978; Floodgate, 1979), and simulated *in situ* experiments involving flow-through seawater tanks (Atlas and Bartha, 1973c), demonstrated that additions of phosphate and nitrate are required for enhanced oil biodegradation in seawater since seawater itself does not usually contain sufficient amounts of these nutrients to sustain continued degradation of oil. In terms of the potential for enhancement, Fusey and Oudot (1973) reported that biodegradation of Kuwait crude oil was increased from 10 to 30% within 17 days by the addition of an organic nutrient mixture containing nitrogen and phosphorus.

The optimum C/N and C/P ratios for petroleum biodegradation in marine waters have been determined in laboratory studies. Bridie and Bos (1971), reported that supplements of 3.2 mg ammonium-N and 0.6 mg phosphorus per liter gave maximum degradation rates for Kuwait crude. In New Jersey coastal waters, Atlas and Bartha (1972b) observed maximum breakdown of Sweden crude by bacteria at nitrogen and phosphorus concentrations of 1 and 0.07 mg per liter, respectively. Reisfield et al. (1972), noted that populations of bacteria in the Mediterranean Sea required 11 mg of nitrogen and 2 mg of phosphorus per liter, to maintain the optimum biodegradation rates for Iranian crude oil. However, nutrients in the form of inorganic salts at concentrations optimized for "closed system" laboratory tests have little or no effect as single applications in the open sea, since they are diluted rapidly and are removed from the oil-water interface where their presence is needed.

In order to maintain optimum nutrient concentrations at the oil-water interface, the use of oleosoluble nitrogen-containing organic nutrients, such as the condensation products of urea and melamine with aldehydes, has been suggested. However, since these organic compounds are soluble in water, they soon leave the oil-water interface and dissipate into the aqueous phase. To overcome this problem attempts have been made to encapsulate the water-soluble nutrients in paraffin (Atlas and Bartha, 1973c; Olivieri et al., 1976), or by generating micro-emulsions of the water-soluble nutrients in a lipophilic phase (Tramier and

Sirvins, 1983).

Atlas and Bartha (1973c) conducted tests in the laboratory under simulated marine conditions to demonstrate the effectiveness of an oleophilic fertilizer consisting of paraffinized urea and octylphosphate in enhancing the biodegradation of oil. The optimal C/N and C/P ratios for oil degradation were found to be 10:1 and 100:1, respectively. In a subsequent field trial, Atlas and Busdosh (1976), showed that within a 21-day period, oleotrophic nitrogen- and phosphorus-containing fertilizers increased the degradation of Prudhoe Bay crude oil in freshwater ponds on the tundra. Furthermore, addition of inorganic nitrogen and phosphorus (as NO_3 and PO_4) did not enhance the degradation of crude oil, contrary to a previous study (Barsdate and Prentki, 1972) which indicated that phosphorus was the limiting nutrient in tundra ponds. Following the preliminary success of their studies, Bartha and Atlas (1976), in conjunction with the U.S. Office of Naval Research, were awarded a patent for the use of fertilizers for stimulating oil-degradation in seawater.

Olivieri et al. (1976), developed a slow release paraffin-supported fertilizer containing $MgNH_4PO_4$ as the active ingredient. The biodegradation of Sarir crude oil in seawater was found to be considerably enhanced by this fertilizer in preliminary experiments. After 21 days, 63% of the crude oil had disappeared when the fertilizer was added as compared to 40% in the control area which recieved no fertilizers. Results of both field and laboratory experiments suggest that paraffin-supported

fertilizers could be used to enhance the degradation of residual oils left behind by mechanical recovery processes.

The main limitation of paraffin-supported fertilizers is due to the use of paraffins as the lipophilizing agent. Paraffinized particles become agglomeration centers, especially when applied to viscous oils, and hence decrease the surface area of the oil available to microbial attack. In addition, paraffin coatings of the fertilizer themselves become an additional contaminant which must be degraded. Finally, the release of nutrients in the paraffin-supported fertilizer is controlled by physical factors (thickness and continuity of the paraffin coat, temperature, etc.), rather than in response to biological demand. In response to the above restrictions, Olivieri et al. (1978) screened a number of inexpensive nitrogen- and phosphorus-containing compounds with intrinsic lipophilic properties in an attempt to find one which was not soluble in water, was stable under environmental conditions, biodegradable, and nontoxic. Laboratory tests with Basra crude oil indicated that soya-bean lecithin and ethyl allophanate met the above criteria and were suitable sources of phosphorus and nitrogen for the oil-degrading microorganisms. Furthermore, in theory enzyme-dependent mechanisms would regulate the release of nutrients from these compounds according to environmental needs. Although preliminary results obtained in the laboratory were extremely positive, field trials to evaluate the effectiveness of these substances to enhance biodegradation of petroleum hydrocarbons in the marine

environment have not been conducted.

Bergstein and Vestal (1978), monitored the degradation of Prudhoe Bay crude oil in arctic tundra ponds fertilized with "Victawet 12", (2-ethylhexyl-dipolyethylene oxide phosphate, Stauffer Chemical Co.), a commercial oleophilic phosphate fertilizer and inorganic phosphate. Bacterial enumeration of water and sediment samples indicated that oil alone did not appear to exhibit either a stimulatory or a toxic effect on the numbers of heterotrophic and oil-degrading bacteria. In contrast, addition of the oleophilic phosphate mixture at concentrations of 0.1 mM, at weekly intervals, significantly stimulated the indigenous microflora in both the presence and absence of oil. Since equal concentrations of inorganic phosphate did not increase the microflora, the stimulation induced by the oleophilic nutrients was attributed to soluble hydrocarbon components within the nutrient mixture. ^{14}C-hydrocarbon mineralization studies demonstrated that the microflora mineralized the saturate fraction of the oil before the polyaromatic fraction. The conclusions obtained in this study support those of Atlas and Busdosh (1976); namely that oleophilic fertilizers may be used to enhance the biodegradation of oil in the aquatic environment.

Tramier and Sirvins (1983), successfully enhanced biodegradation of Arabian light crude oil in temperate and Antarctic waters with EAP 22, (Elf Aquitaine, France), an oleophilic micro-emulsion containing a solution of urea in brine, encapsulated in oleic acid as the external phase, with a laurylphosphate used as a co-

surfactant. The results of experiments conducted in the laboratory with cultures and in large outdoor enclosures with indigeneous microflora were quite encouraging. When acclimated species were involved, temperature did not appear to be a limiting factor for the biodegradation of oil even in Antarctic waters. Calculations from 7 day experiments indicated that the rate of oil loss increased from 10-20 % to 60-85% in waters supplemented with the oleophilic nutrients, even at temperatures ranging as low as 3°C.

Bronchart et al. (1985) recently suggested that oleo-soluble nitrogen-containing organic nutrients may be detrimental to pelagic organisms in the open sea since they may become a barrier to the transfer of air and light into the water column, in a manner similar to the oil slick to which they are applied. In response to this limitation, they developed a new generation of oil dispersants which contain oleophilic nutrients. Theoretically, these new dispersants would dissipate the oil and enhance the development of microbial growth around the dispersed oil droplets, since the nutrients remain at the oil-water interface. The proposed nutrient formulations composed of an oleophilic (normal paraffin or olefin) and a hydrophilic moiety (containing nitrogen and or phosphorus), have the properties of a dispersant as a result of the surfactant-like structure of the nutrient mixtures. Pilot scale trials with Kuwait crude oil in seawater indicated that the "type-B" nutrients (patents pending), containing a nonyl chain and having dodecyl and oleyl radicals as

lipophilic moieties, exhibited high dispersion efficiency and initiated biodegradation of the dispersed crude oil shortly after addition. The nitrogen content in the nutrient formulations was restricted to approximately 1% by the solute properties of the solvents used. However, this did not appear to be a serious problem since it was sufficient to initiate the biodegradation process, and the biomass which developed on the droplets was subsequently sustained by the ambient concentrations of nitrogen in the sea.

In addition to nitrogen and phosphorus, other nutrients may limit the biodegradation of petroleum hydrocarbons in the marine environment. The essential mineral nutrient iron is one of the most abundant elements in the lithosphere (Deevey, 1970), however, its precipitation as ferric hydroxide under alkaline reaction may cause it to limit biological productivity in surface waters in a manner similar to that of phoshorus and nitrate (Sverdrup et al., 1970). Dibble and Bartha (1976), studied the effects of nitrogen, phosphorus, and iron supplements on the biodegradation of South Louisiana crude oil in polluted (10,900 oil-degraders per liter) and relatively clean (750 oil-degraders per liter) littoral seawater samples collected along the New Jersey coast. Without supplements, biodegradation of the crude oil was negligible in both seawater samples. Addition of nitrogen and phosphorus supported rapid biodegradation (72% in 3 days) in the polluted seawater. Total iron in this polluted seawater sample was high (5.2 mM), and the addition of chelated iron did not increase biodegradation rates. However, in the less

polluted seawater sample with lower concentrations of iron (1.2 uM), biodegradation of the crude oil was considerably slower (21% in 3 days), and the addition of chelated iron had a significant stimulatory effect. Although the effect was far less dramatic than that of nitrogen and phosphorus, this experiment conclusively demonstrated that in oligotrophic waters iron may limit petroleum biodegradation. In terms of nutrient enrichment, ferric octoate, an oily substance which was relatively insoluble in water but mixed homogeneously in South Louisiana crude oil was found to be as effective as chelated iron in stimulating the biodegradation of oil. Dibble and Bartha (1976), suggest that surface oil slicks in temperate oligotrophic waters may be treated with a combination of ferric octoate, and paraffin-coated urea and octyl-phosphate.

Contrary to the results obtained in pelagic studies, recent experiments suggest that biodegradation of oil in the littoral zone may be enhanced by water soluble nutrients. Halmo (1985) monitored the degradation of a weathered Statfjord crude oil emulsion in the supralittorial zone on the coast of Norway. The direct addition of commercial-grade water-soluble nutrient fertilizers (NH_4^+ and NO_3^-) or an oil-soluble fertilizer originally developed for use offshore (urea in a microemulsion of oleic acid), were both effective in enhancing natural biodegradation rates. Biodegradation of the oil stranded on vegetation in the superalittoral zone was significantly increased by the application of nutrients. No statistically significant

difference in oil biodegradation was observed between use of the oil-soluble and the water soluble fertilizer. During the initial 4 month period following the spill, in the fertilized areas 45 to 85% of the paraffins in the crude oil were decomposed, while corresponding degradation in the unfertilized reference site was only 10 to 25%. After one year, the n-alkanes were completely degraded in both of the fertilized plots, while 20 to 30% of the paraffins were still present in the reference site. Enhancement in the biodegradation of the oil in the fertilized plots was confirmed by time-series observation of the ratios between normal and branched C-17 and C-18.

During the Baffin Island Oil Spill Project (Eimhjellen et al., 1982, 1983; Eimhjellen and Josefsen, 1984), experimental plots treated with 50% oil-water emulsions of slightly weathered Venezuela Lago Medio crude oil were used to test the effectivenesss of a commercial agricultural fertilizer, Fullgjodsel C (16% N, 6.6% P, 11.9% K, 1.2% Mg, 1.6% S, 3.0% Ca, 0.02% B), to enhance the biodegradation of oil stranded on the Arctic coastline. To mimic the scenario of oil washed ashore at high tide under severe wave action, an experimental site just above the high water swash zone was chosen. Initial experimental results obtained within 2-3 weeks, indicated that bacterial numbers in the plot treated with a low concentration of the fertilizer (40 g/m^2) were elevated an order of magnitude or more above the oiled control plot. In the plot treated with fertilizer at a concentration 10 times higher, there was a corresponding increase in bacterial numbers. Contrary to

expectations, bacterial numbers in the plot with the higher concentration of fertilizer mechanically mixed into the oiled sand were similar to those found in the plot fertilized with the lower concentration of fertilizer. Although analysis indicated that a substantial percentage of the viable bacteria in the oiled control plots were potential oil degraders, even after a 2-year period significant changes in the biodegradation index (ratio of alkanes/isoprenoids) were not observed (Boehm et al., 1984). In contrast, within the fertilized plots degradation of 30-60% of the biologically vulnerable alkane fraction of the oil occurred within one year. Enhanced oil-degradation rates were directly attributable to higher bacterial biomass sustained in the fertilized plots during the experimental period. However, while the numbers of heterotrophic and oil-degrading bacteria suggest that mechanical mixing of the fertilizer into the sediment offered few advantages, analysis of the alkane/isoprenoid ratio indicated that this contention was wrong. A combination of fertilization and mechanical mixing of the oil and fertilizer into the sediment supported optimal biodegradation rates, possibly because of improved penetration of oxygen.

The negative environmental impact of oil in the littoral zone may be reduced by simple nutrient enrichment techniques. Field trials show that the effectiveness of fertilizer treatment in this zone are dependent on geomorphological conditions (Eimhjellen and Josefsen, 1984). However, in fine sediments, an increase of 10-100 times in the levels of oil-degrading bacteria

relative to unfertilized controls has been observed during a 2-year period. Corresponding chemical analyses indicated a 3-5 fold increase in the rate of biodegradation, at least for the alkane fraction of oil. In coarse sediments the effect of the fertilizer appeared to be marginal.

In conclusion, studies indicate that following most oil spill incidents, the available concentrations of nitrogen and phosphorus severely limit the rate and extent of hydrocarbon biodegradation. Natural rates of nutrient replenishment in the marine environment are generally inadequate to support rapid biodegradation of large quantities of oil. There is no doubt that fertilization of isolated spill areas should be seriously considered as a means to enhance microbial degradation. Based on the data available, optimum N:P:C ratios for the degradation of petroleum can be approximated in the field and the addition of appropriate fertilizers can be used to enhance petroleum biodegradation by the indigenous microflora.

5.0. SUMMARY

Despite continuous input, the levels of petroleum hydrocarbons detected in the World oceans is low, presumably because of biodegradative processes. In the short term, biodegradation plays a small role in the dispersal and degradation of petroleum following pollution incidents, but over large temporal and spatial scales, it is probably the major mechanism for

decontamination.

The hydrocarbons in petroleum can be metabolically degraded by numerous species of bacteria, yeasts, fungi and algae. Such microorganisms have been found to be ubiquitous in the World's oceans, and studies have shown that their numbers and degradation potential are generally higher near sources of chronic petroleum input. However, despite the fact that high numbers of oil-degrading microorganisms may be present in the marine environment, natural environmental conditions rarely favour optimal rates of biodegradation.

Our knowledge of the interactive and/or sequential events which occur during microbial degradation of petroleum hydrocarbons is incomplete. We know that a succession of species is involved in the biodegradation of hydrocarbons in nature and a number of rate-limiting factors have been identified. To enhance our predictive capabilities regarding the fate of petroleum hydrocarbons in the environment, the ecological significance of biodegradative mechanisms, such as co-metabolism and the microbial formation of persistant contaminants from petroleum hydrocarbons require further research.

During the last decade, much environmental research has been focussed on the development of applied field techniques which could be used to enhance biodegradation of oil in the environment. In situ field studies have shown that low temperatures alone cannot account for the limited rates of

hydrocarbon biodegradation. Furthermore, field trials have demonstrated that seeding of oil contaminated areas with "oil-degrading" bacteria cultures offers few advantages since they cannot compete against indigenous microflora. Extensive studies concluded that the degradation of petroleum hydrocarbons in the marine ecosystem is limited primarily by oxygen availability and nutritional factors, e.g. nitrogen and phosphorus. Results of preliminary experiments using techniques of oxygenation, chemical dispersion, and nutrient enrichment, to enhance the biodegradation of petroleum, indicate that fertilization with nitrogen and phosphorus offers the great promise as a countermeasure to combat oil spills in the marine environment.

With regard to fertilization as an oil pollution countermeasure, the optimum nutrient concentrations for the degradation of petroleum under diverse marine environments has yet to be determined. Additional laboratory and field experiments are required to assess the effectiveness of inexpensive oleophilic nutrients and their method of application in the open sea. More research is required to evaluate the application of water-soluble nutrients on the intertial zone as a means of enhancing the biodegradation of stranded oil. Furthermore, to improve our predictive capabilities regarding the fate of oil in the marine environment, time-series chemical studies must be initiated to monitor the effect of fertilization on the rate and extent of petroleum hydrocarbon biodegradation.

6.0. REFERENCES

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